

Use of VLCFAE for identifying herbicidally active compoundsField of The Invention

The invention relates to the use of nucleic acids which code for plant polypeptides with the biological activity of very long chain fatty acid elongases and to the use of the polypeptides encoded thereby for identifying novel, herbicidally active compounds, and to methods for finding modulators of these polypeptides.

Background of the Invention

Fatty acid elongases are also referred to as 3-ketoacyl-CoA-synthases or as "condensing enzymes". Very long chain fatty acid elongases, abbreviated to VLCFAE hereinafter, are responsible for the biosynthesis of fatty acids with a chain length of more than 18 C atoms (Millar, A.A. and Kunst, L. 1997, The Plant Journal 12(1), 121-131). The substrates recognized by VLCFAE apart from malonyl-CoA ester are only fatty acid-CoA esters with chain lengths of greater than or equal to C<sub>18</sub> (C<sub>18</sub>+), and the proteins are probably microsomally associated in the cytoplasm. Synthesis of fatty acids with up to 18C atoms *de novo* in plants takes place in the chloroplast, and the fatty acids are esterified by an acyl carrier protein (ACP), (WO 98/54954). Long-Chain Fatty Acids, referred to as VLCFA hereinafter, are components and precursors of the wax layer in the cuticle of plants. They may be components and precursors of cell membranes or constituents of specific storage triacylglycerides. However, they may also be constituents or precursors of lipids with a signal function or with a second messenger function in the plant, such as, for example, the sphingolipids. (Millar, A.A. and Kunst, L. 1997, The Plant Journal 12(1), 121-131, Millar et al. 2000, Trends in Plant Science 5, 95-101).

Detailed Description

Biochemical research (Matthes et al., Z. Naturforsch. 1998, 53c, 1004-1011, Abulnaja and Harwood, Phytochemistry 1991, 30(5), 1445-1447) has revealed that herbicidal substance classes such as, for example, chloroacetamides, oxyacetamides and thiocarbamates inhibit the synthesis of long-chain fatty acids. However, this research did not show whether this takes place by direct inhibition of or interaction with fatty acid elongase enzymes. It is equally unclear which of the many elongases

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also possible, for example by exchange of promoters, for the tissue-specific lipid synthesis to be deliberately changed within one plant species. Diverse publications deal with research of this type. (see, for example, WO 96/13582, WO 98/46766, WO 98/54954, WO 95/15387, WO 00/08172).

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It has now been found, surprisingly, that the *fiddlehead* phenotype can be induced with a herbicidal active substance from the oxyacetamide class, flufenacet. The active substance phenocopies *fiddlehead*. In addition, the active substance also leads to fusion of the rosette leaves.

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The finding that the oxyacetamide flufenacet induces *fiddlehead*-like fusion of flower organs leads to the conclusion that FIDDLEHEAD (FDH), a very long chain fatty acid elongase, is the interaction partner (the target) of the herbicidal active substance.

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Since the VLCFAE, in particular also FIDDLEHEAD from Arabidopsis and other plants, show extensive homologies with one another, it is also possible to use homologous polypeptides encoded by appropriate homologous nucleic acids, and other members of the gene family, as molecular interaction partners (targets) for herbicidal active substances and the aforementioned class of substances. It is thus possible for herbicidal active substances to interact with various VLCFAE, although the interaction with the various VLCFAE occurring in plants may not always have the same strength. This might explain inter alia the observed selectivity of the herbicide class. Other members of the gene family, such as fatty acid elongases and beta-ketoacyl-CoA synthases from Arabidopsis, which are homologous with FIDDLEHEAD (FDH) from *Arabidopsis thaliana* (Acc. No. AJ010713 or O64846) are listed by way of example and not definitively in the following table and in Fig. 1. The degree of phylogenetic relationship between the genes shown can also be inferred qualitatively therefrom.

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	Gene ( <i>Arabidopsis thaliana</i> )	Accession Number	Description
1	<b>T1D16.11</b>	trembl AC004484 AC004484_11	putative beta-ketoacyl-CoA synthase
2	<b>F18A8.1</b>	trembl AC003105 AC003105_1	putative beta-ketoacyl-CoA synthase
3	<b>F16F14.22</b>	trembl AC007047 AC007047_19	putative beta-ketoacyl-CoA synthase
4	<b>F20D22.1</b>	trembl AC002411 AC002411_1	putative 3-oxoacyl-[acyl-carrier-protein] synthase (EC 2.3.1.41)
5	<b>CUT1</b>	trembl AF129511 AF129511_1	"very-long-chain fatty acid condensing enzyme" (CUT1)
6	<b>KCS1</b>	trembl AF053345 AF053345_1	fatty acid elongase 3-ketoacyl-CoA-synthase 1-gene ("KCS1"); KCS1-Product: "fatty acid elongase 3-ketoacyl-CoA synthase 1".
7	<b>AT4g34510</b>	tremblnew AL161585 ATCHRIV81_35	putative ketoacyl-CoA synthase
8	<b>T3A4.10</b>	trembl AC005819 AC005819_10	putative beta-ketoacyl-CoA synthase
9	<b>AT4g34250</b>	tremblnew AL161585 ATCHRIV81_9	fatty acid elongase-like protein
10	<b>AT4g34520 (FAE 1)</b>	tremblnew AL161585 ATCHRIV81_36	fatty acid elongase 1 (FAE 1)
11	<b>F14P13.12</b>	trembl AC009400 AC009400_12	putative fatty acid elongase 3-ketoacyl-CoA synthase 1
12	<b>F4F15.270</b>	trembl AL049711 ATF4F15_27	beta-ketoacyl-CoA synthase-like protein
13	<b>T8O18.8</b>	trembl AC007171 AC007171_8	putative fatty acid elongase

A conclusion in the publication by Böger et al. 2000 (Pest Manag. Sci. 56; 497-508) is that plant VLCFAE are the primary targets of chloroacetamides.

5 However, it is found in the present application for the first time by the example of the FIDDLEHEAD VLCFAE that VLCFAE are protein targets for herbicidal active substances and can be employed to identify novel, improved herbicidal active substances in methods (assays) suitable for this purpose.

10 This applies in particular to polypeptides from plants showing a homology to the FIDDLEHEAD (FDH) polypeptide from Arabidopsis, preferably encompassing

polypeptides with a homology of 60%, particularly preferably of 80% and very particularly preferably of 90%, to FDH.

5 Particular preference is given in this connection to FDH-homologous VLCFAE from Arabidopsis for identifying novel, herbicidal active substances, preferably encompassing polypeptides with a homology of 60%, particularly preferably of 80% and very particularly preferably of 90%, to FDH.

10 It is possible in a very particularly preferred manner for the FDH polypeptide from Arabidopsis to be used to identify novel herbicidal active substances.

The present invention therefore relates to the use of plant polypeptides with the biological activity of a VLCFAE for identifying VLCFAE modulators.

15 The present invention likewise relates to the use of plant polypeptides with the biological activity of a VLCFAE having a homology of 60%, preferably of 80% and particularly preferably of 90% to the FDH polypeptide from Arabidopsis for identifying VLCFAE modulators.

20 The present invention likewise relates to the use of polypeptides from Arabidopsis with the biological activity of a VLCFAE which have a homology of 60%, preferably of 80% and particularly preferably of 90% to the FDH polypeptide from Arabidopsis for identifying VLCFAE modulators.

25 The present invention relates in particular to the use of the FIDDLEHEAD polypeptide having SEQ ID NO: 2 for identifying VLCFAE modulators.

The present invention likewise relates to the use of nucleic acids encoding for VLCFAE for identifying VLCFAE modulators.

The present invention likewise relates to the use of nucleic acids coding for VLCFAE and having a homology to the fiddlehead sequence according to a coding sequence of SEQ ID NO: 1 of 60%, preferably of 80% and particularly preferably of 90% for identifying VLCFAE modulators. Homologues of Arabidopsis are particularly preferred in this connection.

The present invention relates in particular also to the use of the nucleic acid coding for the FIDDLEHEAD polypeptide and having SEQ ID NO: 1 for identifying VLCFAE modulators.

The polynucleotides or polynucleotide fragments to be used according to the invention are preferably those corresponding to polynucleotides from plants.

The polynucleotides or polynucleotide fragments to be used according to the invention are particularly preferably those corresponding to polynucleotides from Arabidopsis.

The polypeptides to be used according to the invention very particularly preferably include a sequence selected from

- a) the sequence shown in SEQ ID NO: 2,
- b) sequences encoded by a nucleic acid having SEQ ID NO: 1,
- c) partial sequences of the sequences defined under a) or b) which still have the biological activity of a VLCFAE,
- d) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under a) to c),

- e) sequences which include the C-terminally localized active site of the polypeptide shown in SEQ ID NO: 2,
- f) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under e),
- g) sequences which include the specific N terminus of the polypeptide shown in SEQ ID NO: 2, and
- h) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under g).

A very particularly preferred embodiment of the nucleic acids to be used according to the invention is represented by a cDNA molecule having the FDH-encoding sequence shown in SEQ ID NO: 1, the introns shown in SEQ ID NO: 1 no longer being present.

The FDH-encoding regions of the nucleic acid shown in SEQ ID NO: 1 extend from position 176-583, 1119-1745 and 1821-2438.

The nucleic acids to be used according to the invention are, in particular, single-stranded or double-stranded deoxyribonucleic acids (DNA) or ribonucleic acids (RNA). Preferred embodiments are fragments of genomic DNA, which may contain introns as shown in SEQ ID NO: 1 and cDNAs.

The nucleic acids to be used according to the invention are preferably DNA or DNA fragments corresponding to genomic DNA from plants.

The nucleic acids to be used according to the invention are particularly preferably DNA or DNA fragments corresponding to genomic DNA from Arabidopsis.

The nucleic acids to be used according to the invention very particularly preferably include a sequence selected from

- a) the sequence shown in SEQ ID NO: 1,
- b) sequences which code for a polypeptide including the amino acid sequence shown in SEQ ID NO: 2,
- c) partial sequences at least 14 base-pairs long of the sequences defined under a) or b),
- d) sequences which hybridize to the sequences defined under a) or b),
- e) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under a) or b),
- f) sequences which code for the C-terminally localized active site of the polypeptide shown in SEQ ID NO: 2
- g) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under f), –
- h) sequences which code for the specific N terminus of the polypeptide shown in SEQ ID NO: 2,
- i) sequences which have an identity of at least 60%, preferably of 80%, particularly preferably of 90%, with the sequences defined under h),
- j) sequences which are complementary to the sequences defined under a) to i), and



- k) sequences which, because of the degeneracy of the genetic code, code for the same amino acid sequence as the sequences defined under a) to h).

5 A very particularly preferred embodiment of the nucleic acids to be used according to the invention is represented by a cDNA molecule with the FDH-encoding sequence shown in SEQ ID NO: 1.

10 The term "to hybridize" as used herein describes the process in which a single-stranded nucleic acid molecule enters into base pairing with a complementary strand. It is possible in this way, starting from the sequence information disclosed herein, for example to isolate DNA fragments from plants other than Arabidopsis which code for VLCFAE and, in particular, for FIDDLEHEAD, which have the same or similar properties of an elongase with the amino acid sequence shown in SEQ ID NO: 2. Thus, for example, the nucleic acid sequence coding for the N-terminal polypeptide  
15 sequence characteristic of FDH can be used in order to find nucleic acid sequences and polypeptides which are sequentially and functionally homologous to FDH and are suitable for identifying novel herbicidal active substances.

Hybridization conditions are calculated approximately by the following formula:

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The melting temperature  $T_m = 81.5^{\circ}\text{C} + 16.6 \log [c(\text{Na}^+)] + 0.41(\% \text{ G} + \text{C}) - 500/n$   
(Lottspeich and Zorbas, 1998).

25 In this,  $c$  is the concentration and  $n$  is the length of the hybridizing sequence section in basepairs. The term  $500/n$  is omitted for a sequence of  $>100$  bp. With the highest stringency, washing is carried out at a temperature of  $5-15^{\circ}\text{C}$  below  $T_m$  and an ionic strength of  $15 \text{ mM Na}^+$  (equivalent to  $0.1 \times \text{SSC}$ ). If an RNA probe is used for the hybridization, then the melting point is  $10-15^{\circ}\text{C}$  higher.

30 Preferred hybridization conditions are indicated below:

Hybridization solution: DIG Easy Hyb (supplied by Roche) Hybridization temperature: 37°C, preferably 42°C (DNA-DNA), 50°C (DNA-RNA).

1. washing step: 2x SSC, 0.1% SDS 2x5 min at room temperature;
- 5 2. washing step: 1x SSC, 0.1% SDS 2x 15 min at 50°C; preferably 0.5x SSC, 0.1% SDS 2x 15 min at 65°C; particularly preferably 0.2x SSC, 2x15min at 68°C.

10 The term “active site” refers to a peptide region which functionally corresponds to the peptide region with an amino acid sequence from position 191 to position 310 of the sequence shown in SEQ ID NO: 2. The reactive amino acid cystein is located at position 257.

15 The degree of identity of the nucleic acids is preferably determined with the aid of the NCBI BLASTN program, version 2.0.4. (Altschul et al., 1997).

20 The present invention also relates to the use, in methods for identifying VLCFAE inhibitors, of the regulatory regions which, in plant cells, especially in Arabidopsis, naturally control the transcription of the nucleic acids to be used according to the invention.

25 The term “regulatory regions” as used herein refers to nontranslated regions of the relevant gene, such as promoters, enhancers, repressor or activator binding sites or termination sequences, which interact with cellular proteins, leading to control of transcription.

30 The present invention further relates to the use of DNA constructs which include a nucleic acid to be used according to the invention and a heterologous promoter for identifying VLCFAE modulators.

The term "heterologous promoter" as used herein refers to a promoter which has properties different from those of the promoter which controls expression of the relevant gene in the original organism.

5 The selection of heterologous promoters depends on whether prokaryotic or eukaryotic cells or cell-free systems are used for expression. Examples of heterologous promoters are the 35S promoter of cauliflower mosaic virus for plant cells, the alcohol dehydrogenase promoter for yeast cells, the T3, T7 or SP6 promoters for prokaryotic cells or cell-free systems, and tissue-specific promoters  
10 from plants, for example the epidermis-specific promoter of FIDDLEHEAD and the promoters of other tissue-specific elongases.

The present invention further relates to the use of vectors which contain a nucleic acid to be used according to the invention, a regulatory region to be used according  
15 to the invention or a DNA construct to be used according to the invention, in methods for identifying the VLCFAE modulators.

Vectors which can be used are all phages, plasmids, phagemids, phasmids, cosmids, YACs, BACs, artificial chromosomes or particles suitable for particle bombardment  
20 which are used in molecular biology laboratories.

Preferred vectors are pBIN (Bevan, 1984) and its derivatives for plant cells, pFL61 (Minet et al., 1992) or, for example, the p4XXprom vector series (Mumberg D., Müller R. and Funk M. 1995, Gene 156, 119-122) for yeast cells, pBLUESCRIPT-  
25 vectors for bacterial cells, lamdaZAP (from Stratagene) for phages.

The present invention also relates to the use of host cells which contain a nucleic acid to be used according to the invention, a DNA construct to be used according to the invention or a vector to be used according to the invention, for identifying VLCFAE  
30 modulators.

The term "host cell" as used herein refers to cells which do not naturally contain the nucleic acids to be used according to the invention and are suitable for expression of the polypeptide to be used according to the invention.

- 5        Suitable host cells are both prokaryotic cells, preferably *E. coli*, and eukaryotic cells such as cells of *Saccharomyces cerevisiae*, *Pichia pastoris*, insects, plants, frog oocytes and mammalian cell lines.

- 10        The term "polypeptides" as used herein refers both to short amino acid chains, which are usually referred to as peptides, oligopeptides or oligomers, and to longer amino acid chains which are usually referred to as proteins. It encompasses amino acid chains which may be modified either by natural processes, such as post-translational processing, or by chemical methods which are state of the art. Such modifications may occur at various points and a plurality of times in a polypeptide, such as, for  
15        example, on the peptide backbone, on the amino acid side chain, on the amino terminus and/or on the carboxyl terminus. They comprise, for example, acetylations, acylations, ADP ribosylations, amidations, covalent linkages with flavins, haem portions, nucleotides or nucleotide derivatives, lipids or lipid derivatives or phosphatidylinositol, cyclizations, formation of disulphide bridges, demethylations, cystine formations, formylations, gamma-carboxylations, glycosylations,  
20        hydroxylations, iodinations, methylations, myristoylations, oxidations, proteolytic processings, phosphorylations, selenoylations and tRNA-mediated additions of amino acids.

- 25        The peptides according to the invention may be in the form of "mature" proteins or in the form of parts of larger proteins, for example as fusion proteins. They may furthermore have secretion or "leader" sequences, prosequences, sequences which make simple purification possible, such as multiple histidine residues, or additional stabilizing amino acids.

The polypeptides to be used according to the invention need not be complete VLCFAE but may also be only fragments thereof as long as they still have a biological activity of the complete VLCFAE. Polypeptides which exercise a biological activity of the same type as a VLCFAE with an amino acid sequence as shown in SEQ ID NO: 2 are also regarded as being according to the invention. The polypeptides to be used according to the invention moreover need not correspond to the VLCFAE from Arabidopsis. Also regarded as polypeptides to be used according to the invention are polypeptides which are homologous to VLCFAE for example of the following plants or to fragments thereof which can still exercise the biological activity of the latter:

Dicotyledonous plants of the genera: Abutilon, Amaranthus, Ambrosia, Anoda, Anthemis, Aphanes, Atriplex, Bellis, Bidens, Capsella, Carduus, Cassia, Centaurea, Chenopodium, Cirsium, Convolvulus, Datura, Desmodium, Emex, Erysimum, Euphorbia, Galeopsis, Galinsoga, Galium, Hibiscus, Ipomoea, Kochia, Lamium, Lepidium, Lindernia, Matricaria, Mentha, Mercurialis, Mullugo, Myosotis, Papaver, Pharbitis, Plantago, Polygonum, Portulaca, Ranunculus, Raphanus, Rorippa, Rotala, Rumex, Salsola, Senecio, Sesbania, Sida, Sinapis, Solanum, Sonchus, Sphenoclea, Stellaria, Taraxacum, Thlaspi, Trifolium, Urtica, Veronica, Viola, Xanthium.

Dicotyledonous plants of the genera: Arachis, Beta, Brassica, Cucumis, Cucurbita, Helianthus, Daucus, Glycine, Gossypium, Ipomoea, Lactuca, Linum, Lycopersicon, Nicotiana, Phaseolus, Pisum, Vicia.

Monocotyledonous plants of the genera: Aegilops, Agropyron, Agrostis, Alopecurus, Apera, Avena, Brachiaria, Bromus, Cenchrus, Commelina, Cynodon, Cyperus, Dactyloctenium, Digitaria, Echinochloa, Eleocharis, Eleusine, Eragrostis, Eriochloa, Festuca, Fimbristylis, Heteranthera, Imperata, Ischaemum, Leptochloa, Lolium, Monochoria, Panicum, Paspalum, Phalaris, Phleum, Poa, Rottboellia, Sagittaria, Scirpus, Setaria, Sorghum.

Monocotyledonous plants of the genera: Allium, Ananas, Asparagus, Avena, Hordeum, Oryza, Panicum, Saccharum, Secale, Triticale, Triticum, Zea.

5 The polypeptides to be used according to the invention may, by comparison with the corresponding region of naturally occurring VLCFAE, have deletions or amino acid substitutions as long as they still exercise the biological activity of the complete fatty acid elongases. Conservative substitutions are preferred. Such conservative substitutions comprise variations where one amino acid is replaced by another amino acid from the following group:

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1. small aliphatic residues which are nonpolar or of low polarity: Ala, Ser, Thr, Pro and Gly;
2. polar, negatively charged residues and their amides: Asp, Asn, Glu und Gln;
3. polar, positively charged residues: His, Arg and Lys;
- 15 4. large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. aromatic residues: Phe, Tyr und Trp.

The following list shows preferred conservative substitutions:

Original residue	Substitution
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val

Original residue	Substitution
Lys	Arg
Met	Leu, Tyr, Ile, Phe
Phe	Met, Leu, Tyr, Trp
Ser	Thr
Thr	Ser
Trp	Tyr, Phe
Tyr	Trp, Phe
Val	Ile, Leu

The present invention thus also relates to the use of polypeptides which exercise the biological activity of a VLCFAE and comprise an amino acid sequence which shows at least 60% identity, preferably 80% identity, particularly preferably 90% identity and particularly preferably 97-99% identity with the sequence shown in SEQ ID NO: 2 over the entire length thereof.

The degree of identity of the amino acid sequences is preferably determined with the aid of the BLASTP + BEAUTY programme, version 2.0 4. (Altschul et al., 1997).

A preferred embodiment of the polypeptides to be used according to the invention is the VLCFAE FIDDLEHEAD (FDH) with an amino acid sequence as shown in SEQ ID NO: 2.

The FDH amino acid sequence belongs on the basis of homology (Yephremov et al. 1999) to the family of fatty acid elongases (FAE). The FDH protein has two potential transmembranes domains in the region of the amino acids from about 53 to 75 and in the region from about 93 to 115.

The first 45-50 amino acids at the N terminus are specific for the FIDDLEHEAD protein whose biological role has not as yet been fully elucidated.

The N terminus is so specific that it can be used as recognition sequence for FDH.

5 The term "biological activity of a VLCFAE" as used herein means the ability to biosynthesize fatty acids with a chain length of 18 and more C atoms, and to the recognition of the substrates malonyl-CoA esters and/or fatty acid CoA esters with chain lengths greater than or equal to C<sub>18</sub>.

10 The nucleic acids to be used according to the invention can be prepared in the conventional way. For example, the nucleic acid molecules can be completely chemically synthesized. It is also possible for short pieces of the nucleic acids according to the invention to be chemically synthesized and for such oligonucleotides to be labelled radioactively or with a fluorescent dye. The labelled oligonucleotides can also be used to screen cDNA banks produced starting from plant mRNA. Clones to which the labelled oligonucleotides hybridize are selected for isolating the relevant DNA fragments. After characterization of the isolated DNA,  
15 the nucleic acids to be used according to the invention are obtained in a simple manner.

20 The nucleic acids to be used according to the invention can also be prepared by PCR methods using chemically synthesized oligonucleotides.

25 The term "oligonucleotide(s)" as used herein means DNA molecules consisting of 10 to 50 nucleotides, preferably 15 to 30 nucleotides. They are chemically synthesized and can be used as probes.

30 The polypeptides to be used according to the invention which are encoded by the nucleic acids according to the invention can be prepared by cultivating, under suitable conditions, host cells which contain nucleic acids to be used according to the invention. The desired polypeptides can then be isolated in a conventional way from the cells or the culture medium. The polypeptides can also be prepared in *in-vitro* systems.



A rapid method for isolating the polypeptides to be used according to the invention which are synthesized by host cells using a nucleic acid to be used according to the invention starts with the expression of a fusion protein where the fusion partner can be affinity-purified in a simple manner. The fusion partner can be, for example, glutathione S-transferase. The fusion protein can then be purified on a glutathione affinity column. The fusion partner can be removed by partial proteolytic cleavage for example at linkers between the fusion partner and the polypeptide according to the invention which is to be purified. The linker can be designed so that it includes target amino acids, such as arginine and lysine residues, which define sites for trypsin cleavage. Such linkers can be generated by employing standard cloning methods using oligonucleotides.

Further possible purification methods are based on preparative electrophoresis, FPLC, HPLC (for example using gel filtration, reverse phase or slightly hydrophobic columns), gel filtration, differential precipitation, ion exchange chromatography and affinity chromatography.

Since VLCFAE are membrane proteins or membrane-associated proteins, detergent extractions are preferably carried out during the purification methods, for example using detergents which affect the secondary and tertiary structures of the polypeptides only slightly or not at all, such as nonionic detergents.

Purification of the polypeptides to be used according to the invention may encompass the isolation of membranes starting from host cells which express the nucleic acids according to the invention. Such cells preferably express the polypeptides to be used according to the invention in an adequate copy number so that the amount of polypeptides in a membrane fraction is at least 10 times higher than that found in comparable membranes of cells which express the FDH gene naturally; the amount is particularly preferably 100 times, very particularly preferably at least 1000 times, higher.

For assay purposes it is also possible for a membrane fraction, in particular a microsome fraction, to be concentrated and used without special purification.

5 The terms "isolation or purification" as used herein mean that the polypeptides to be used according to the invention are separated from other proteins or other macromolecules of the cell or of the tissue. A composition containing the polypeptides to be used according to the invention is preferably concentrated at least 10-fold and particularly preferably at least 100-fold in terms of the protein content by comparison with a preparation from the host cells.

10 The polypeptides to be used according to the invention can be affinity-purified even without fusion partners, with the aid of antibodies which bind to the polypeptides.

15 The present invention also relates to methods for finding chemical compounds which bind to the polypeptides according to the invention and alter their properties. Because of the diverse functions of the VLCFAE according to the invention, modulators which influence the activity may represent novel growth-regulating or herbicidal active substances. Modulators may be agonists or antagonists, or inhibitors or activators.

20 The term "agonist" as used herein refers to a molecule which promotes or enhances the VLCFAE activity.

25 The term "antagonist" as used herein refers to a molecule which retards or impedes the VLCFAE activity.

30 The term "modulator" as used herein represents the generic term for agonist and antagonist. Modulators may be small organic chemical molecules, peptides or antibodies which bind to the polypeptides according to the invention. Modulators may furthermore be small organic chemical molecules, peptides or antibodies which bind to a molecule which in turn binds to the polypeptides according to the invention

and thus influences their biological activity. Modulators may be natural substrates and ligands or structural or functional mimetics thereof.

The modulators are preferably small organic chemical compounds.

Binding of the modulators to the VLCFAE may alter the cellular processes in a way leading to death of the plants treated therewith.

The present invention therefore also relates to VLCFAE modulators which have been found with the aid of a method described above for identifying modulators of the FDH protein or of a VLCFAE homologous thereto.

The present invention further relates to methods for finding chemical compounds which alter the expression of the polypeptides to be used according to the invention. Such “expression modulators” may also represent novel growth-regulating or herbicidal active substances. Expression modulators may be small organic chemical molecules, peptides or antibodies which bind to the regulatory regions of the nucleic acids coding for the polypeptides to be used according to the invention. Expression modulators may furthermore be small organic chemical molecules, peptides or antibodies which bind to a molecule which in turn binds to regulatory regions of the nucleic acids coding for the polypeptides to be used according to the invention, and thus influences expression thereof. Expression modulators may also be antisense molecules.

The present invention likewise relates to the use of modulators of the polypeptides to be used according to the invention or of expression modulators as plant growth regulators or herbicides.

The present invention likewise relates to expression modulators of VLCFAE which are found with the aid of the method described above for finding expression modulators.

The methods according to the invention include high-throughput screening (HTS). It is possible to use for this both host cells and cell-free preparations which contain the nucleic acids to be used according to the invention and/or the polypeptides to be used according to the invention.

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In order to find modulators it is possible to incubate a synthetic reaction mix (for example products of *in vitro* transcription) or a cellular constituent, such as a membrane or any other preparation which contains the polypeptides to be used according to the invention, together with a labelled substrate or ligand of the polypeptides in the presence and absence of a candidate molecule which may be an agonist or antagonist. The ability of the candidate molecule to increase or inhibit the activity of the polypeptides to be used according to the invention is evident from an increased or reduced binding of the labelled ligand or from an increased or reduced conversion of the labelled substrate. Molecules which bind well and lead to an increased activity of the polypeptides according to the invention are agonists. Molecules which bind well and inhibit the biological activity of the polypeptides to be used according to the invention are good antagonists. These may be inhibitors of the abovementioned classes of herbicidal substances without, however, being restricted thereto.

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Detection of the biological activity of the polypeptides to be used according to the invention can be improved by a so-called reporter system. Reporter systems in this regard comprise, but are not restricted to, colorimetrically labelled substrates which are converted into a product, or a reporter gene which responds to changes in the activity or the expression of the polypeptides to be used according to the invention, or other known binding assays.

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The activity of membrane-associated proteins can advantageously be measured in another way. Functional heterologous expression of such proteins in *E. coli* is often difficult or impossible. In this case it is possible by suitable cloning (for example using suitable PCR strategies) to separate the catalytically active part of the protein

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from the membrane-associated part of the protein so that the gene product represents a soluble protein and can easily be purified. A wide repertoire of measurement possibilities is available for measuring the activity of soluble proteins. Particularly sensitive measurement can take place for example by fluorescence polarization with use of a fluorescence-labelled ligand or substrate.

Another example of a method with which modulators of the polypeptides to be used according to the invention can be found is a displacement assay in which, under conditions suitable for this purpose, the polypeptides to be used according to the invention and a potential modulator are compacted with a molecule which is known to bind to the polypeptides to be used according to the invention, such as a natural substrate or ligand or a substrate or ligand mimetic. The substrate or ligand mimetic may be, for example, radioactively labelled or provided with a fluorescent or dye marker (also UV). The polypeptides to be used according to the invention themselves can also be labelled in an analogous way so that the number of polypeptides which are bound to a ligand or have undergone a reaction can be determined accurately. It is possible in this way to estimate the efficacy of an agonist or antagonist.

The invention further relates to the use of a nucleic acid to be used according to the invention, of a DNA construct to be used according to the invention or of a vector to be used according to the invention for producing transgenic plants, and to the corresponding transgenic plants as such or parts or propagation material thereof.

The present invention likewise relates to transgenic plants, parts of plants, protoplasts, plant tissues or plant propagation materials in which, after introduction of a nucleic acid to be used according to the invention, of a DNA construct to be used according to the invention or of a vector to be used according to the invention, the intracellular concentration of VLCFAE is increased or reduced compared with the corresponding wild-type forms.

The term "parts of plants" as used herein means all epigeal and hypogeal parts and organs of the plants, such as shoot, leaf, flower and root, and protoplasts and tissue cultures produced therefrom.

- 5 The term "propagation material" as used herein means vegetative and generative propagation material such as cuttings, tubers, rhizomes, layers and seeds.

10 The invention also relates to plants in which alterations are made in the sequence coding for VLCFAE leading to production of a VLCFAE which leads to an increase or reduction in the biological activity or the amount of the VLCFAE present in the plants.

15 The term "mutagenesis" as used herein refers to a method for increasing the spontaneous mutation rate and thus for isolating mutants. This may entail generation of mutants with the aid of mutagens *in vivo*, for example with chemical compounds or physical effects which are suitable for inducing mutations (for example base analogues, intercalating substances, UV rays etc.). The required mutants can be obtained by selection for a particular phenotype. The position of the mutations on the chromosomes can be determined relative to other known mutations by  
20 complementation and recombination analyses. Mutations can also be introduced in a targeted manner into chromosomal or extrachromosomal DNA (*in vitro* mutagenesis, site directed mutagenesis, error-prone PCR etc.).

25 The term "mutant" as used herein refers to an organism which harbours an altered (mutated) gene. A mutant is defined by comparison with the wild-type which harbours the unaltered gene.

30 Since the VLCFAE to be used according to the invention, in particular the FDH protein, plays a part in signalling and development processes, transgenic "sense" plants or plants which have been selected deliberately for an increased amount or activity of corresponding endogenous VLCFAE or elements linked thereto in the

- 5 signal transduction acquire increased resistance to herbicidally active compounds. It is also possible by mutating the amino acid sequence to develop VLCFAE which are resistant to compounds having herbicidal activity on these proteins. Transfer and expression of the genes of such naturally isolated or synthetically produced mutants in plants, especially in crop plants such as, for example, maize, wheat, barley, oats, rice, rye, tomatoes, legumes, potato plants, *Lactuca sativa*, Brassicaceae, wood plants, *Physcomitrella patens*, makes them herbicide-resistant or characterized by increased tolerance to herbicides.

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## Examples

### **Example 1**

Herbicide treatment of *Arabidopsis thaliana* with Flufenacet: generation of the  
5 *fiddlehead* phenotype

*Arabidopsis* seeds (ecotype Columbia Col-0), which were stored at 4°C, were sown in soil and cultivated at 22°C ± 2°C under long-day conditions (16 h light/8h dark).

10 Plants 2 weeks old were sprayed with 250 g/ha and 50 g/ha flufenacet and cultivated further at 22°C ± 2°C under long-day conditions (16 h light/8h dark). Between day 7 and day 11 after flufenacet treatment, the flower organs fuse with the uppermost cauline leaves of the *Arabidopsis* plants. These fusions are very similar to those in the "fiddlehead" mutants. From day 18, all the flowers appear normal again because  
15 flufenacet is degraded by the glutathione S-transferase activity of the plants.

### **Example 2**

Isolation of the described FDH nucleotide sequence

20 Isolation of the described FIDDLEHEAD (FDH) nucleotide sequence can take place in analogy to Yephremov et al. 1999, The Plant Cell 11, 2187-2201, Pruitt et al. 2000, PNAS 97(3), 1311-1316 or as described in WO 98/54954 (clone EL 4). The sequence can also be obtained straightforwardly by methods known to the skilled person with the assistance of the sequence described in SEQ ID NO: 1.

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### **Example 3**

Production of transgenic *Arabidopsis* plants resistant to active substances directed  
against VLCFAE

30 Selected, mutated VLCFAE gene constructs are cloned into suitable pBIN vector constructs and transferred to suitable agrobacteria strains. The vector sequences



harbour a marker gene which can be selected in plants and which confers, for example, resistance to kanamycin or resistance to the herbicide BASTA. *Arabidopsis* plants are grown and, after 4-6 weeks, shortly before flowering, immersed in a suspension of the agrobacteria strain transformed with the vector construct. Contact of the plant tissue with the bacteria suspension can be enhanced by vacuum infiltration. The plants treated in this way are incubated further until the seeds are ripe. The seeds are harvested and placed on selection medium. The only seedlings to survive are those containing the transferred gene construct and the marker gene, those which have thus been successfully transformed. A detailed description of the method can be found in Bechthold N. and Pelletier G.: *In planta Agrobacterium* mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. In: *Arabidopsis Protocols*, edited by Martinez-Zapater J.M. and Salinas J., 1998 Humana Press ISBN 0-89603-391-0.

#### Explanation of the figures and of the sequence listing

**Fig. 1:** Phylogenetic tree of *Arabidopsis thaliana* beta-ketoacyl-CoA synthases with homology to FIDDLEHEAD (FDH) (DNA Star Program MegAlign (Clustal method with PAM250 "residue weight table")).

**SEQ ID NO: 1:** Nucleic acid sequence coding for the "FIDDLEHEAD" (FDH) polypeptide from *Arabidopsis thaliana*, a very long chain fatty acid elongase. This is a genomic sequence, which is why introns are still present. The coding regions are indicated.

**SEQ ID NO: 2:** Amino acid sequence of the "FIDDLEHEAD" (FDH) polypeptide from *Arabidopsis thaliana*.

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